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Production of Bioethanol by Co-Culture of *Bacillus Substilis*, *Gliocladium roseum* and *Saccharomyces cerevisiae* from Sago Waste

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Abstract

Biochemical characterization of sago industry waste shows that it is rich in carbohydrates, in more specific it contains 52 % of starch. This work deals with the management of waste for bioethanol production. The co fermentation to produce ethanol was carried out with *Bacillus substilis* (Bacteria), *Gliocladium roseum* (Fungus) and *Saccharomyces Cerevisiae* (Yeast). The results show that the ethanol yield 19.08 g/L was obtained in the co-culture of *Bacillus substilis, Gliocladium roseum* and *Saccharomyces Cerevisiae.*

Keywords

Sago waste, bioethanol, co-culture, *Gliocladium roseum, Saccharomyces Cerevisiae*.

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INTRODUCTION

In India, cassava (*Manihot esculenta* Crantz) is grown largely over 3.9 million hectares producing 60×10^6 million tons of tubers annually. Cassava is an industrial crop for the production of sago, vermicelli, and starch, whereas each ton of cassava tuber processed for sago and starch, yields half a ton of fibrous residue as waste. Cassava waste contains starch (~50% dry weight), cellulose, hemicellulose, and ashes in the extractable materials. Using cassava waste for ethanol production offers huge opportunities owing to the enormous availability of this inexpensive raw material in the cassava growing countries [1]. Lots of emphasis has been given to screen feasible bioprocess methodologies for efficient conversion of cassava waste to fuel ethanol. Since, starch derived from any plant sources is a complex molecule, therefore, it requires various hydrolytic enzymes to be converted into simple fermentable sugars. A number of strategies have been adopted for the construction of starch-utilizing systems, which include the addition of amylolytic enzymes in culture broth, as well as mixed-culture fermentation.

Conversion of both cellulosic and starchy materials in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. Fungal co-culturing offers a

means to improve hydrolysis of residues, and also to enhance biomass utilization which would minimize the need for additional enzymes in the bioconversion process. Co-cultures have many advantages compared to their monocultures, including improved productivity, adaptability, and substrate utilization [2]. Bioethanol production was studied by Zhang *et.al* [3] using *Aspergillus niger* and *Saccharomyces Cerevisiae* in simultaneous saccharification and fermentation. This work has demonstrated the production of significant quantity of ethanol by a co-culture of *Bacillus substilis* (Bacteria), *Gliocladium roseum* (Fungus) and *Saccharomyces Cerevisiae* (Yeast).

MATERIALS AND METHODS

Collection of Microorganisms

Pure cultures of i) *Gliocladium roseum* (ii) *Bacillus subtilis* and (iii) *Saccharomyces Cerevisiae* which are common lignocelluloses degrading microorganisms, were collected from NCL, Pune, India and maintained as per the supplier instructions at 30°C. They were cultured, sub-cultured and maintained in slants. The characteristics of the above organisms were also studied before using them for enzymatic hydrolysis.

Collection of sago waste

The waste has been collected from a cassava sago industry in Salem. The waste was taken in the solid form that called as Residual pulp (Thippi). Collected sample were dried in sun light and stored at 4 ^{0}C in the Refrigerator.

Biomass collection

The sago waste was washed and dried at atmospheric temperature (28 ± 2 °C) for 3 days. The dry biomass was further ground with an electric blender, filtered with a 60-Mesh (0.250 mm) sieve and stored under dry conditions until use

Biochemical characterization

The method described by Milne et al., [4] was used to determine the dry matter, acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents of the cassava waste. Crude protein was determined by Kjel-dahl method and total carbohydrate by Clegg Anthone
method as described by Sluiter et al., [5].

(a) Cellulose

The ADF was used for the estimation of cellulose employing the method described by Sluiter et al., [5]. The contents of the crucible were covered with cooled (15 °C) 75 % or 24N H_2SO_4 and stirred with glass rod to a smooth paste, breaking all lumps. Thereafter, the crucible was filled about half-full with acid, left to stand for 1h after which the acid was drained away. The crucible was then refilled with 72 % acid and left to stand for 3 h. The acid was filtered off as much as possible with vacuum and the content dried at 100 °C overnight and the weight determined thereafter. The loss in weight was taken as cellulose and it was calculated by the following formula:

$$\text{ADF}(\%) = \frac{\text{Weight of ADF - Weight of dried residue after acid treatment}}{\text{Weight of sample}} \text{ X } 100$$

(b) Hemicellulose

Hemicellulose was determined as the difference between neutral detergent fibre NDF (%) and ADF (%)

Hemicellulose (%) = NDF (%) – ADF (%)

(c) Lignin

The residue that remained after the determination of cellulose was treated with phosphate-buffered solution (0.1 M, KH_2PO_4:K_2HPO_4, pH = 8) of 25 mM $KMnO_4$ for 90 min at 20–25 °C. Lignin was dissolved leaving cutin and silica as insoluble materials. The contents were then filtered through tarred sintered crucible using gentle suction and the residue obtained, washed with distilled water and then with acetone. The crucible and residue were dried in an oven at 100 °C

$$\text{Lignin}(\%) = \frac{\text{Wt. after acid treatment - Wt. after KMnO4 treatment}}{\text{Weight of sample}} \text{ X } 100$$

Sago waste pretreatment process

The sago waste was pretreated using the steam explosion (SE), acid and alkali pretreatment methods. The pretreatment was performed to delignify the sago, a necessary procedure towards liberating cellulose and hemicellulose prior to hydrolysis [6]. SE pretreatment method described by Sharma et al., [7] was employed for the pretreatment of the sago waste. Ten grams of each bio-mass was suspended in 90 mL of distilled water in a conical flask and placed in an autoclave for 45 min at 121 °C. After 45 min, the autoclave was depressurized by suddenly fully opening the valve. The solid residue remaining was collected and extensively washed with tap water until neutral pH was reached prior to simultaneous saccharification and fermentation (SSF). After the solid residue was washed, drying was achieved at 60 °C overnight using the method described by Fan et al., [8]. Thereafter, the dry hydrolysate was analysed for cellulose, hemicellulose and lignin content and stored in sterile polypropylene bags for further use. The acid and alkali pretreatment methods described by Olanbiwoninu and Odunfa [9] were employed in the pretreatment of the sago waste.

Bioethanol production through SSFC of steam exploded sago waste

SSFC was carried out on pretreated sago waste by consortium. The fermentation protocol was carried out under optimal conditions. Four percent (w/v) (i.e. 8 g of sample sago in 200 mL) of the pretreated sample in 250-mL Erlenmeyer flask containing 200 mL of the fermentation medium was used. The medium was sterilized at 121 °C for 20 min at 15 psi. After cooling to room temperature, 4 % (v/v) of each of the inoculum from a 24-h broth culture was added to the suspension of the biomass and incubated at 37 °C. The fermentation broth was monitored daily for 7 days for pH changes, enzyme activity and bacterial growth.

Estimation of fermentation products using gas chromatography–mass spectrometry (GC–MS)

GC-MS Specification:

Before and after treatment of sago waste was analyzed by Gas Chromatography and Mass Spectrometer. The fermentation broth sample was centrifuged to separate suspended particles and the clear liquid was analysed for the presence of fermentation products.

Specifications are given below: **GC Programme:** Column: Elite-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30 x 0.25mm x 0.25µm df**,** Equipment: GC Clarus 500 Perkin Elmer, Carrier gas: 1ml per min, Split: 10:1, Detector: Mass detector Turbo mass gold-Perkin Elmer**,** Software: Turbomass 5.2**,** Sample injected: 2µl. **Oven temperature Programme -** 110° C -2 min hold, Up to 200° C at the rate of 10 ° C/min-No hold, Up to 280 ° C at the rate of 5° C / min-9 min hold, Injector temperature 250° C, Total GC running time 36 min. **MS Programme:** Library used NIST Version-Year 2005,Inlet line tem1perature 200° C, Source temperature 200 ° C, Electron energy: 70 eV, Mass scan (m/z): 45-450,Solvent Delay: 0-2 min, Total MS running time: 36 min

RESULTS AND DISCUSSION

Starch is the major constituent in the waste of sago industry. Because, Cassava is the tuber crop which is one of the supplement of starch and Sago production. Cassava sago waste consists 55-60% of starch [10]. It has negligible amount of Lignin, Hemi cellulose and reducing sugars and it were estimated by suitable methods [11]. Table 1 shows the biochemical composition of cassava sago waste.

Composition analysis and pretreatment

The total carbohydrate and lignin composition of the sago before and after pretreatment is shown in Table. The result indicated that best pretreatment method (steam explosion) achieved increase in total carbohydrate from 70 % to 79 % dry weight while the lignin was reduced from 4.2% to 3.6 %.

GC-MS alalysis

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. In the GC-MS analysis, 13 bio active compounds were identified in the pretreated sago waste. Among the 13 components identified, Phytol is the major component available in 100% with RT 13.88. Another component available RT 12.76 with 64.82%, is Myo-Inositol, 4-C-methyl- ($C_7H_{14}O_6$), which has anti-diabetic activity. Other components like l-Gala-l-ido-octose (RT 8.70), n-Hexadecanoic acid (RT 12.58), Methyl tetradecanoate (RT 12.76), 2, 4, 3, 5-Dimethylene-l-iditol (RT 14.92) etc., available in the raw sago waste sample. GC-MS chromatogram of Sago waste before treatment shown in **Fig.1**

Production of bioethanol by simultaneous saccharification and fermentation

Table 2 shows the summary of the GC–MS estimation of the steam-exploded Sago waste and the fermentative bacteria in consortium. GC–MS chromatogram of the fermentation broth is given in **Fig. 2**. An ethanol content of 19.08 g/L was obtained

in the co-culture of the *Bacillus substilis, Gliocladium roseum* and *Saccharomyces Cerevisiae.*

The processed sago was subjected to three different pretreatments, namely: steam explosion, acid and alkali pretreatments. The choice of the final pretreatment method used for the fermentation process was based on the analysis of the total carbohydrate content and the enzyme productivity obtained after the different pretreatments. The pretreatment method that gave the highest total carbohydrate was steam explosion. The highest carbohydrate content obtained after the pretreatments of the sago was 79 %.

Pretreatment is a necessary step in the use of ligno-cellulosics for bioethanol production. Joshi et al. [12] described pretreatment as the most important rate limiting step in the overall bioethanol production process. Pretreatment was carried out to break the lignin–hemi-cellulose–pectin complex, disrupt/loosen-up the crystalline structure of cellulose and increase the porosity of the biomass used in the study. When these changes are achieved enzymatic saccharification becomes easier, resulting in higher fermentable sugar levels [13-15]. The pretreatment methods employed achieved high delignification of the different agricultural biomass.

Table.1 Biochemical composition of cassava sago waste

Parameters	% Dry Weight
Starch	52
Cellulose	13.6
Lignin	4.2
Hemicellulose	11.8
Reducing sugars	1.8

Table 2: Products of SSF of Sago as obtained from GC-MS analysis of fermentation broth

Sample	Acetone (g/L)	Ethyl acetate (g/L)	Ethanol (g/L)	n-Propanol (g/L)	Isobutanol (g/L)	Acetic acid (g/L)
Sago with co-culture	**3.92**	**8.44**	**19.80**	**5.69**	**5.14**	**6.67**

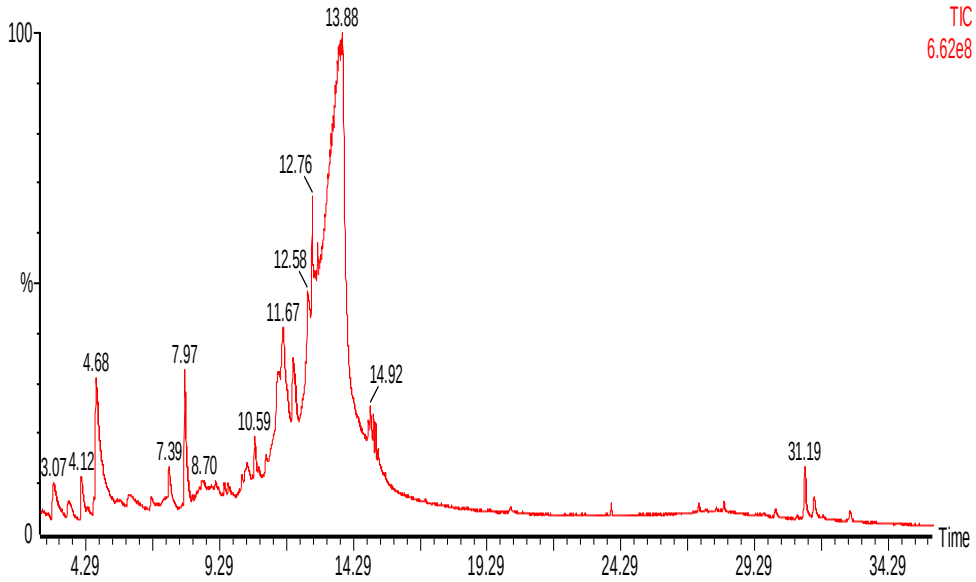

Fig.1: GC-MS chromatogram of Sago waste before treatment

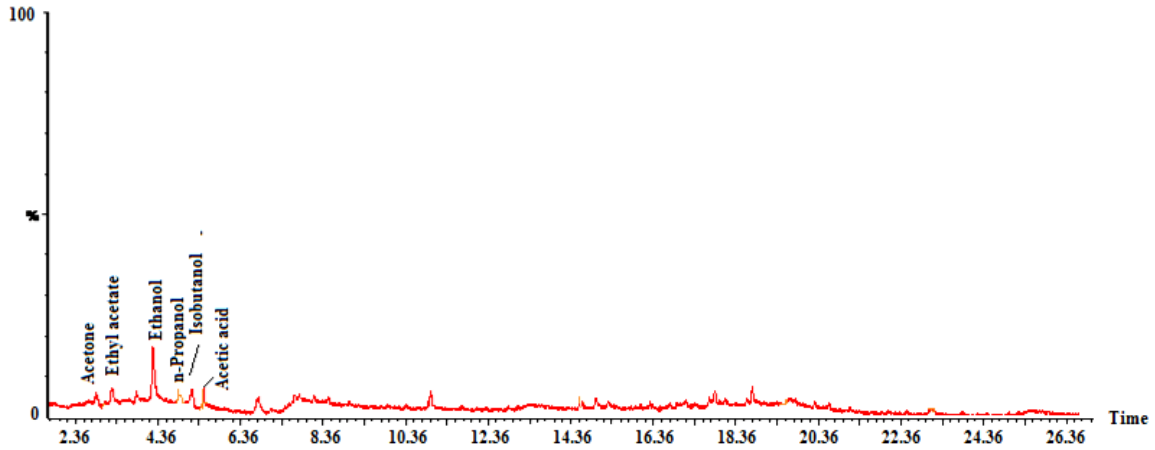

Fig. 2: Chromatogram of fermentation broth showing ethanol production by co-culture of *Bacillus substilis, Gliocladium roseum* and *Saccharomyces Cerevisiae*.with sago as substrate

CONCLUSION

A maximum ethanol yield (19.80 g/L) determined by GC–MS analysis of the fermentation broth after 7 days was obtained in the set-up containing co-culture of *Bacillus substilis* (Bacteria), *Gliocladium roseum* (Fungus) and *Saccharomyces Cerevisiae* (Yeast) sago as the substrate. This study is novel as it has demonstrated efficient ethanol production by co-culture through simultaneous saccharification and co-fermentation of steam-exploded sago. The result reveals that sago waste can be a better alternative substrate for the production of ethanol in industries.

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